Micropropagation of diploid and tetraploid *Plectranthus amboinicus* (Lour.) Spreng

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Abstract. *Plectranthus amboinicus* (Lour.) Spreng is commonly consumed by people in North Sumatra, Indonesia, as a vegetable, which is commonly called Torbangun. Plant tissue culture is an alternative method for the mass-production of transplants. Genetic improvement by somatic cell manipulation such as induction of polyploid plants is needed to improve biomass production as well as their secondary metabolite products. This research aimed to conduct *in vitro* micropropagation of diploid and tetraploid *P. amboinicus* on Murashige and Skoog (MS) medium containing BAP or Kinetin. Shoot tips were used as explants and cultured on MS medium containing BAP or Kinetin at 0, 0.5, 1 and 2 mg/l given as single concentration. The experiment used a completely randomized design with four replications. Each replication consisted of three explants. The results showed that height of shoots was affected by type of cytokinins. Kinetin at 0.5 mg/l gave the highest shoots of tetraploid plants significantly different with addition of BAP both to diploid and tetraploid plants. The number of multiple shoots and leaves varied in both diploid and tetraploid plants. Root formation was best on the medium without addition of cytokinins. Both diploid and tetraploid plantlets had a high survival rate after acclimatization in the greenhouse.

1. Introduction

Plectranthus amboinicus (Lour.) Spreng. is a medicinal vegetable plant. According to ITIS *(Integrated Taxonomic Information System*), this plant is also named as *Coleus amboinicus* (Lour.) [1]. This plant contains some metabolite products which is useful for facilitating the secretion of breast milk. In Indonesia, especially in North Sumatra, *P. amboinicus* (torbangun, bangun-bangun) leaves are consumed by mothers after giving birth and nursing mothers because the leaves of this plant can increase milk production. Consuming the leaves of this plant that contains lactogogum can increase milk production more than consuming supplements facilitating breast milk [2,3]. On the other hand, *P. amboinicus* contains iron, zinc and potassium higher compared to milk supplements. Babies whose mothers consume the leaves of this plant grow faster and healthier [2]. *Plectarnthus amboinicus* leaves contain lactogogum (10-50%), nutrients (10-25%), and pharmaceutics (10-30%) [4] and contain minerals such as calcium, which is high enough to be beneficial in accelerating growth and development of baby's bones [5].

Until now, research on *P. amboinicus* mostly been carried out in pharmacology [6], product development from *P. amboinicus* leaves [7,8], functional drinks [9] and nutritional studies [4]. Efforts to increase growth and production of the plants have been carried out by application of organic fertilizer, with water hyacinth compost fertilizer and pruning [10,11]. Tissue culture

research has been conducted, including micropropagation of this plant in culture media containing 2,4-D and BAP [12,13], also in media containing BAP, Kinetin and NAA [14] and increased *in vitro* plant growth [15]. Effect of cytokinin in combination with elicitors was also investigated for micropropagation, metabolite production and molecular evaluation [16]. Very limited research has been done on genetic improvement to produce superior plant varieties.

Genetic improvement of *P. amboinicus* can be done, among others, by somatic cell manipulation using polyploidization techniques to produce tetraploid plants. Polyploid plants, including tetraploid, have thicker leaves, greener leaf colors, and larger stem and root diameters, greater flower size, and a rougher leaf surface than diploids [17-22]. However, polyploidy can also cause shorter plants [18], and different leaf shapes compared to diploid plants [18,23,24]. But on average polyploid plants are larger and higher biomass production than diploid plants [25-27]. Tetraploid plants have four sets of chromosomes. Changes in the number of chromosomes will change the morphology, anatomy, and physiology of plants, thereby increasing genetic diversity. Polyploid plants can occur naturally or artificial. One of the artificial polyploid induction techniques that is often used is by using chemicals such as colchicine and oryzalin [28].

To confirm the ploidy level, the common analysis used is by flow cytometer. This technique is widely used for determining ploidy levels of plants because it can analyze faster and easier. The ploidy level is determined by measuring the relative total fluorescence intensity of DNA from each plant cell, so it does not require large number of samples [29]. Analysis using a flow cytometer can detect ploidy levels with large populations, faster, more accurate as well as easier to detect mixoploids or aneuploidies [30]. Ploidy level confirmation using flow cytometer has been widely used in various plants such as *Artemisia annua* [31], Pamelo [32], taro [33,34], bananas [35], and guava [36], and water spinach plant [37].

Plectranthus amboinicus tetraploid was produced by induction of *in vitro* plants by oryzalin (unpublished data). The use of anti-mitotic compounds such as colchicine and oryzalin for doubling chromosomes has been carried out in many *Coleus* species or Lamiaceae family for example at *Lippia alba* [38] and *Tetradenia riparia* [39]. Although colchicine is more efficient in producing polyploid plants, colchicine is very toxic to humans. Oryzalin is less toxic than colchicine [40]. Colchicine has a weak affinity for plant tubulin, so that to induce polyploid plants must be used at millimolar concentrations, while oryzalin has a strong affinity for plant tubulin so it only requires lower concentrations (in micromolar) to induce polyploid plants [41,42]. The aim of this research was to conduct *in vitro* micropropagation of diploid and tetraploid *P. amboinicus* on Murashige & Skoog (MS) medium containing BAP and Kinetin.

2. Materials and methods

2.1 Shoot multiplication

Plant materials used in this research were *in vitro* shoot tips of *P. amboinicus* diploid and tetraploid cultured on Murashige & Skoog (MS) medium [43] without the addition of plant growth regulators according to Noorrohmah [18]. Eight-week-old *in vitro* shoot tips at about 1-2 cm long were planted on the treatment MS medium containing BAP or Kinetin at a concentration of 0 (control), 0.5, 1, and 2 mg/l. Each treatment consisted of four replications (bottles) containing three shoots. The medium was supplemented with 30 g/l of sugar and solidified with 3.5 g/l of agar (Gelzan). The pH of the media was adjusted to 5.8, and then the media was sterilized using an autoclave at 121ºC and a pressure of 1 atm for 15 min. The shoot cultures were maintained in the incubation room at temperature 25-26°C with continuous lighting (light intensity of 1000 lux) for eight weeks. Plant growth observations, including the measurement of the height of shoots, shoot numbers, leaf numbers, and root numbers, were done once a week. Data at week 8 were analyzed using analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) at level of 5%.

2.2 Confirmation of ploidy level

Ploidy level analysis of *P. amboinicus* was performed using a BD Accuri $C6^+$ flow cytometer (USA) following the method for *Artemisia annua* L., according to Hafiizh *et al.* [44]. The plant material used was *in vitro* diploid and tetraploid leaves at eight weeks. Leaves at about 0.5 cm^2 were placed on a petri dish, dripped with 250 µl of Nuclei Extraction Buffer, then chopped using a razor blade until smooth. Subsequently, the fine leaves were filtered with 30 µm pore sized celltrics, the filtrate was then put into a cuvette tube, then added with 500 µL Staining Solution, Propidium Iodide and RNAse for analysis. Diploid plant leaves were used as a control. The average DNA content and coefficient of variation (CV) of each tetraploid sample at each peak were recorded, then compared with diploid plant peak as control.

2.3 Acclimatization

Twelve weeks old of diploid and tetraploid plantlets cultured on plant growth regulator free MS medium (MS0) were taken out from bottles. Only plants grown on this medium formed root. The roots were washed from the remaining agar carefully. Plantlets with drained roots were then planted in plastic pots containing mixed soil, compost and roasted husks (1:1:1) and maintained in the greenhouse. Each pot contained one plant, there were 20 diploid plantlets as well as 20 tetraploids to have 40 pots in total. After watering, the pot was covered with transparent plastic for about two weeks until new leaves appeared. Plants were watered every day. The survived plants were observed and presented as the percentage of survived plants twelve weeks after acclimatization.

2.4 Stem diameter and leaf area measurements

Stem diameter and leaf area of *P. amboinicus* was measured on acclimated plants at 12 weeks of age. Stem diameter was measured on the third internodes from the shoot tips using calipers. The number of samples measured was from 20 diploid as well as 20 tetraploid plants, taken from 10 different pots. The leaves measured are the leaves that have the largest width in a pot. The leaf area measurement was done using millimeter block paper. The leaf sampled from 10 different plants of diploid and tetraploid were stuck to the millimeter block paper. The pattern was drawn according to the shape and size of the leaf. The number of millimeter boxes in the leaf pattern then was calculated as leaf area.

2.5 Data analysis

This research used a completely randomized design. Each treatment consisted of four replications (bottles), where each bottle contained three explants. Data of growth eight weeks after planting were analyzed using Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) at 5% significance level.

3. Results and discussion

3.1 Growth of diploid and tetraploid shoot cultures in vitro

The growth of diploid and tetraploid shoots of *P. amboinicus* for one to eight weeks is shown in Figure 1. The addition of Kinetin in the medium increased the height of shoot compared to the addition of BAP. In diploid plants the shoot height in the medium containing BAP was the lowest (Figure 1A), while in media containing higher Kinetin, an increase in growth occurred from four weeks after planting (4 WAP) (Figure 1B). Shoot height in tetraploid plants on MS media containing BAP and Kinetin increased in weeks 3 to 7 (Figures 1C and D). At eight weeks, there was no increase in growth. Media containing 0.5 mg/l Kinetin was the best medium for tetraploid shoot height growth. Thus, the addition of Kinetin at low concentrations can stimulate the height of tetraploid shoots. The same finding occurred in teak tissue culture that 0.75 mg/l Kinetin increased the growth of teak shoots. While the higher Kinetin, which was 1.5 mg/l inhibited shoot growth. The higher the Kinetin concentration the shorter the height of shoots [45]. Similarly, the addition of 0.5 mg/l Kinetin on *P.*

amboinicus gave 30% shoots formation initiated from apical shoots gave 30% while from lateral shoots gave 40% shoot initiation after addition with 5 mg /l Kinetin [14].

Kinetin is a cytokinin type that is having more influence on shoot development processes. The energy needed for elongation of shoots is used to form multiple shoots to inhibit the shoot height. Cytokinin such as Kinetin plays a role in cell division only, so Kinetin suppresses bud height [45]. Cytokinin stimulates cell division and inhibits elongation so that multiple shoots are formed, shoot elongation is inhibited [46].

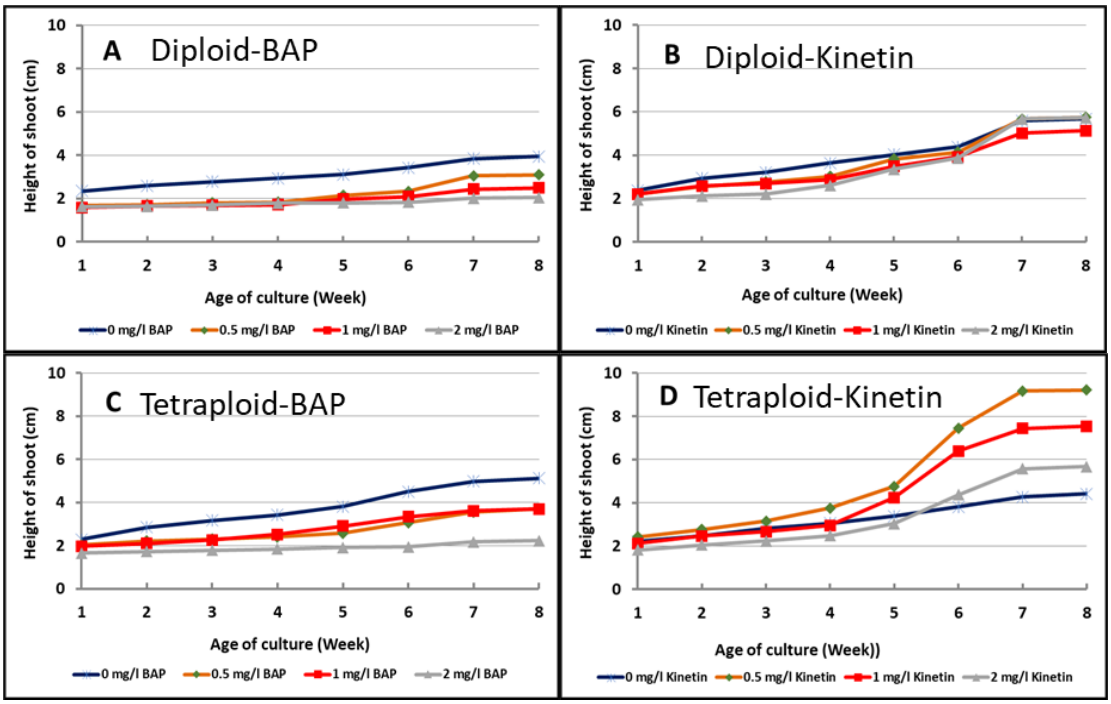

Figure 1. Height of *Plectranthus amboinicus* shoots cultured for 1-8 weeks. Diploid plants cultured on MS medium containing BAP (A), Kinetin (B), and tetraploid plants on MS medium containing BAP (C), Kinetin (D).

The results showed that the addition of BAP and Kinetin to MS media increased the number of shoots of diploid and tetraploid *P. amboinicus* started from beginning of culture until eight weeks of culture (Figure 2). In diploid plants, the addition of BAP (Figure 2A) increased the number of shoots higher than the addition of Kinetin (Figure 2B) from 2-3 weeks to eight weeks. Growth of diploid shoots number on MS medium containing BAP increased from two weeks to eight weeks after culture. In MS medium containing Kinetin, the number of shoots increased from week-4 to week-8 of culture. The growth of *P. amboinicus* tetraploid shoots (Figures 2C and D) was faster than that of diploid shoots.

Unlike in diploid plants, in tetraploid plants, MS medium containing BAP increased shoots numbers occurred from weeks-3 to week-8 (Figure 2C). In MS medium containing Kinetin, the number of tetraploid shoots increased from week-2 to week-8 of culture. The highest number of shoots was found in tetraploid plants on MS media containing 2 mg/l BAP (Figure 2C). This is similar with the results reported by Rahman [14] that the highest number of shoots was by the addition of BAP but not with Kinetin. Thus, it has been proven that the type of plant growth regulator influences the formation of multiple shoots in *P. amboinicus* genotype.

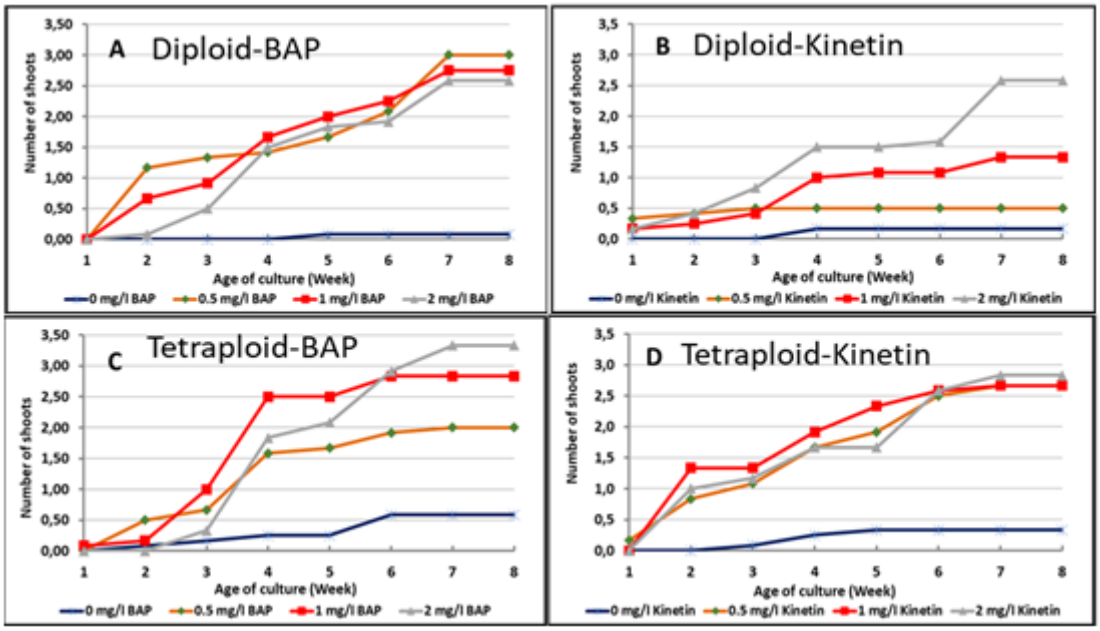

Figure 2. Number of *Plectranthus amboinicus* shoots cultured for 1-8 weeks. Diploid plants cultured on MS medium containing BAP (A), Kinetin (B), and tetraploid plants on MS medium containing BAP (C), Kinetin (D).

Figure 3 shows the growth of diploid and tetraploid *P. amboinicus* leaf numbers at one to eight weeks after culture. The results showed that the addition of BAP or Kinetin increased the growth of diploid and tetraploid plant leaves depending on the type of cytokinin and its concentration. In MS media containing BAP, the number of leaves of diploid plants increased from week-3 until week-8 after culture (Figure 3A). In MS media containing Kinetin an increase in the number of leaves occurred from week-4 to week-8 after culture (Figure 3B). In tetraploid plants, leaf numbers in MS media with the addition of BAP started to increase from weeks-4 to week-8 (Figure 3C), while in MS media containing Kinetin number of leaves started to increase from week-2 to week-8 of culture (Figure 3D). In taro cultivars Bolang and Pontianak, the higher Kinetin concentration, the fewer leaves were formed [47].

Growth of root numbers of *P. amboinicus* from one to eight weeks after culture on MS media containing BAP and Kinetin is shown in Figure 4. The results showed that the growth of diploid plants differed from tetraploid. The addition of BAP significantly inhibited root growth compared to the addition of Kinetin to both diploid and tetraploid plants. The number of roots of diploid plants on MS medium without BAP, increase from week-1 to week-4. However, on MS media with the addition of BAP, root growth very slowly from the beginning of culture until week-4, and increase until week-7 (Figure 4A). In MS medium without Kinetin, roots of diploid plants grew faster (Figure 4B). The addition of 0.5 mg/l Kinetin increased the number of roots until week-7, in contrary 2 mg/l Kinetin resulted in prolonged growth of roots (Figure 4B). The number of roots of tetraploid plants in MS medium with BAP addition was very slow (Figure 4C). After seven weeks of culture, there was no increase in the number of roots (Figures 4C and D). The addition of 0.5 and 1.0 mg/l Kinetin increased root growth of tetraploid plants after six weeks of culture higher than root growth on the medium without the addition of Kinetin (Figure 4D). Commonly, high Kinetin concentration has a negative effect on plant development [48]. Similarly, addition of BAP at high concentrations also inhibits shoot growth on explants so that the percentage of shoot and root formation decreases [49].

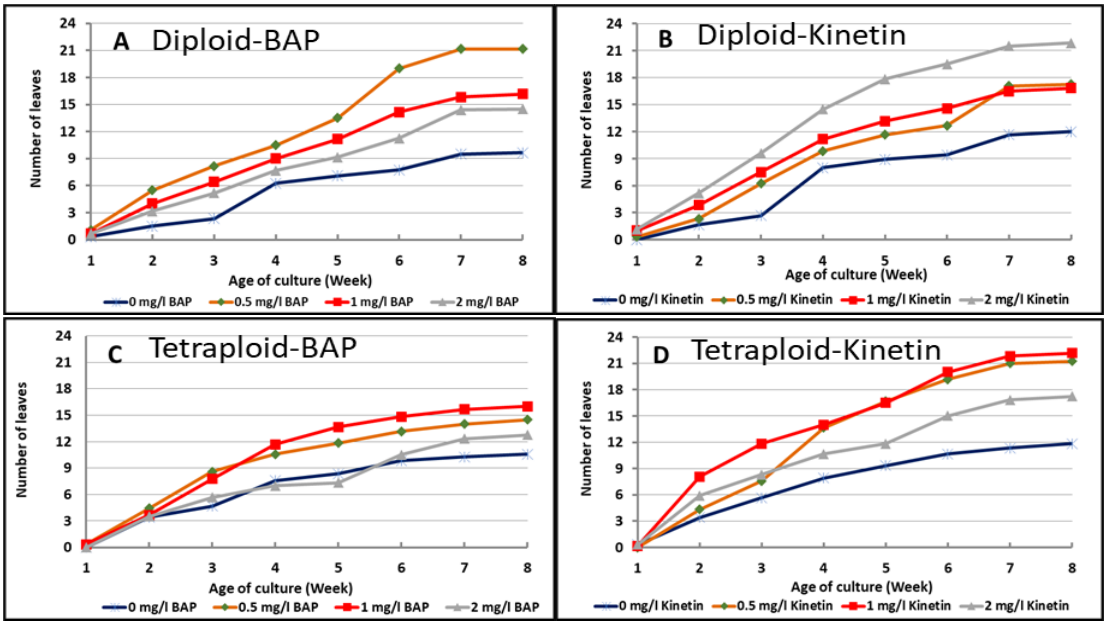

Figure 3. Leaf number of *Plectranthus amboinicus* shoots cultured for 1-8 weeks. Diploid plants cultured on MS medium containing BAP (A), Kinetin (B), and tetraploid plants on MS medium containing BAP (C), Kinetin (D).

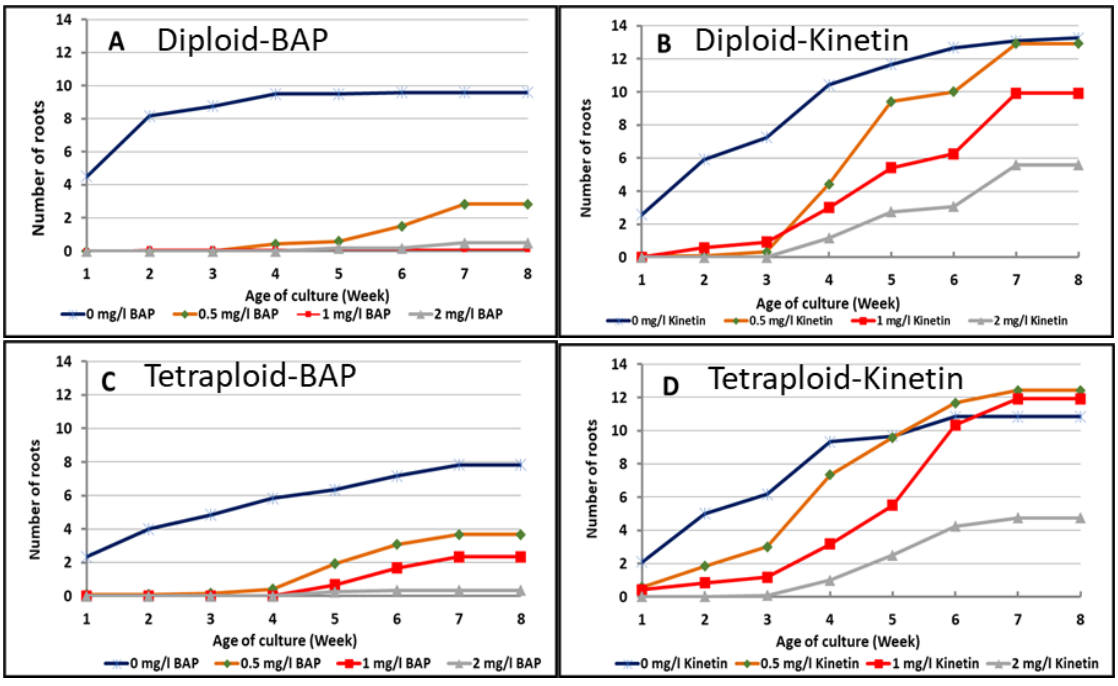

Figure 4. Root numbers of *Plectranthus amboinicus* shoots cultured for 1-8 weeks. Diploid plants cultured on MS medium containing BAP (A), Kinetin (B), and tretraploid plants on MS medium containing BAP (C), Kinetin (D).

Table 1 shows analysis of variance of different plant genotypes and type of cytokinins on the growth of *P. amboinicus* after eight weeks of culture. The result indicated that the genotype (diploid and tetraploid) factor significantly affected shoot height and shoot numbers but not affected to leaf and root numbers. Cytokinin types (BAP and Kinetin) factor, as well as genotype vs cytokinins type factors, affected all growth parameters observed. In taro shoot culture, the number of leaves and the number of roots decreases with the addition of high Kinetin concentration [47].

Table 1. Anova of different clone and type cytokinins treatment (BAP and Kinetin) on growth parameters of *Plectranthus amboinicus in vitro* shoots after 8 weeks of treatment.

No	Growth parameters	F value & Significanty			CV (%)
		Genotype	Cytokinin Type	Genotype vs Cytokinin Type	
1.	Shoot height	13.16**	224.7**	3.82**	39.16
2.	Shoot numbers	8.79**	18.28**	3.41**	65.66
3.	Leaf numbers	0.22^{ns}	10.13**	2.99**	35.57
4.	Root numbers	0.06^{ns}	135.70**	3.91**	30.47

Notes : * : significant on α: 5%; ** : highly significant; ns : not significant.

Table 2. Average of shoot length, shoot numbers, leaf numbers and root numbers of *in vitro* diploids and tetraploids *Plectranthus amboinicus at* eight weeks after planting on the media containing different types of cytokinins.

Clone	Cytokinin (mg/l)	Shoot length (cm)	Shoot numbers	Leaf numbers	Root numbers
Diploid	0 BAP	3.95 ± 0.15^{def}	0.08 ± 0.08^{e}	9.67 ± 0.64^{g}	9.58 ± 0.69^{c}
	0.5 BAP	3.10 ± 0.43^{efg}	3.00 ± 0.49^{ab}	21.17 ± 2.28^{abc}	2.83 ± 0.47^{g}
	1 BAP	2.50 ± 0.26^{fg}	2.75 ± 0.46^{ab}	16.17 ± 2.32^{cdef}	**0.08 ± 0.08^{h}**
	2 BAP	2.05 ± 0.14^{g}	**2.58 ± 0.42^{ab}**	14.50 ± 2.28^{defg}	**0.50 ± 0.26^{h}**
	0 Kinetin	5.67 ± 0.64^{c}	0.17 ± 0.17^{e}	12.00 ± 0.82^{defg}	**13.25 ± 0.61^{a}**
	0.5 Kinetin	5.75 ± 0.65^{c}	0.50 ± 0.23^{de}	17.25 ± 1.74^{abcd}	**12.92 ± 0.57^{a}**
	1 Kinetin	5.13 ± 0.74^{cd}	1.33 ± 0.33^{cd}	16.83 ± 2.07^{bcdef}	9.92 ± 1.03^{c}
	2 Kinetin	5.73 ± 0.50^{c}	2.58 ± 0.23^{ab}	21.83 ± 1.03^{ab}	5.58 ± 0.38^{e}
Tetraploid	0 BAP	5.13 ± 0.38^{cd}	0.58 ± 0.23^{de}	10.58 ± 0.78^{g}	7.83 ± 0.58^{d}
	0.5 BAP	3.70 ± 0.24^{defg}	2.00 ± 0.21^{bc}	14.50 ± 0.99^{defg}	3.67 ± 0.54^{fg}
	1 BAP	3.70 ± 0.51^{defg}	2.83 ± 0.42^{ab}	16.00 ± 1.67^{cdef}	2.33 ± 0.81^{g}
	2 BAP	2.23 ± 0.11^{g}	3.33 ± 0.26^{a}	12.75 ± 1.05^{defg}	0.33 ± 0.19^{h}
	0 Kinetin	4.41 ± 0.36^{cde}	0.33 ± 0.19^{de}	11.83 ± 1.34^{defg}	10.83 ± 0.96^{bc}
	0.5 Kinetin	9.21 ± 0.90^{a}	2.67 ± 0.58^{ab}	21.25 ± 1.89^{abc}	12.42 ± 0.50^{ab}
	1 Kinetin	7.54 ± 0.45^{b}	2.67 ± 0.28^{ab}	22.17 ± 1.36^{a}	11.92 ± 0.56^{ab}
	2 Kinetin	5.68 ± 1.01^{c}	**2.83 ± 0.61^{ab}**	17.25 ± 2.30^{abcde}	4.75 ± 0.51^{ef}

Notes: Number followed with the same letters on the same column is not significantly different according to Duncan Multiple Range Test at α = 5%.

Table 2 shows the average value of shoot height, shoot numbers, leaf numbers and root numbers of diploids and tetraploids *P. amboinicus in vitro* culture after eight weeks of treatment with different types of cytokinins. The results indicated that after eight weeks in culture, tetraploid plant had higher shoot length, shoot number and leaf number, but not root number compared to diploid plant. Polyploid

(including tetraploid) plants have more number of chromosomes than diploid plants, therefore, tetraploid plants look more muscular, the parts of the plant become larger (roots, stems, leaves, flowers, and fruits), the cells (clearly visible on the epidermis) is larger, the cell nucleus is also larger, the transport reeds have a larger diameter, and the stomata are larger [50] The highest shoot was found on tetraploid plants grown on MS medium containing 0.5 mg/l Kinetin, and the highest shoot number was on MS medium containing 2 mg/l BAP.

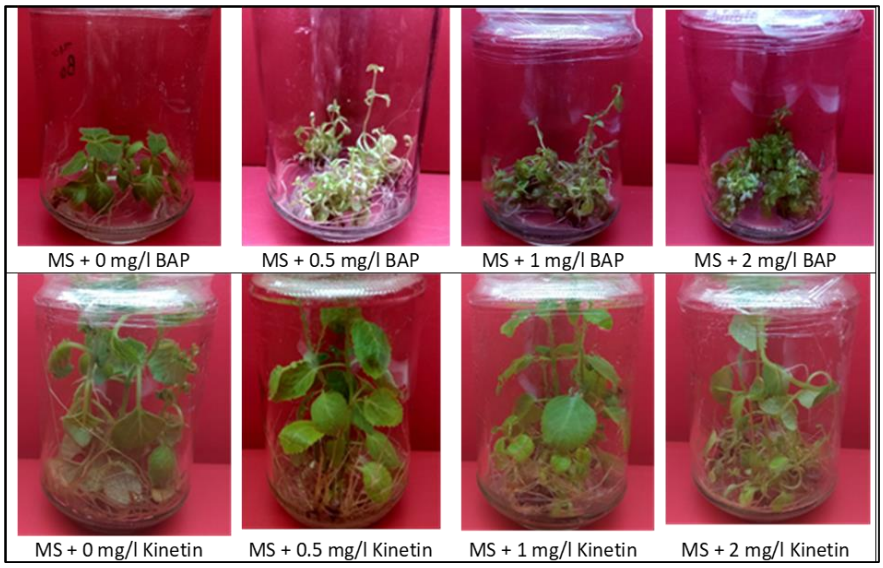

Figure 5. Performance of *in vitro* diploids *Plectranthus amboinicus* after eight weeks cultured on control MS containing BAP and Kinetin at 0, 0.5, 1 and 2 mg/l.

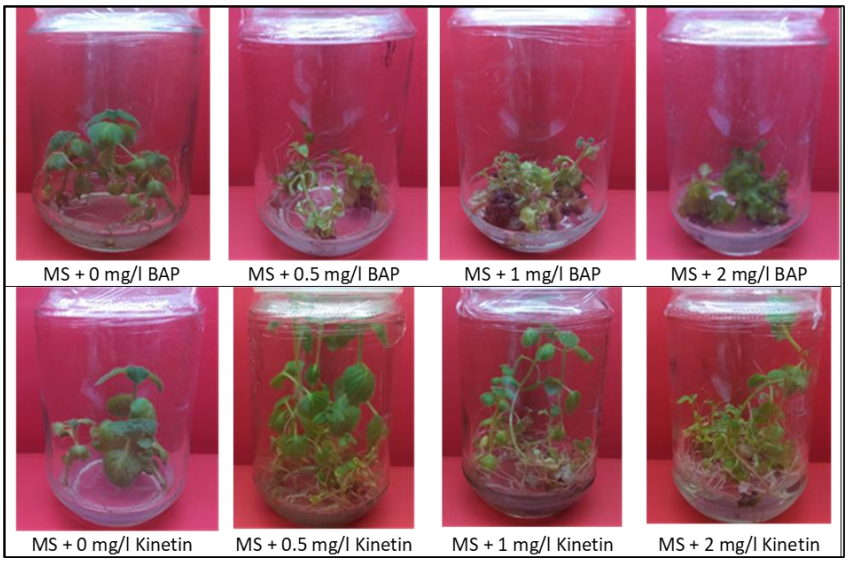

Figure 6. Performance of *in vitro* tetraploids *Plectranthus amboinicus* after eight weeks cultured on control MS containing BAP and Kinetin at 0, 0.5, 1 and 2 mg/l.

The highest number of leaves of diploid plants were found in MS media containing 2 mg/l Kinetin, but nor significantly different with that in media containing 0.5 and 1 mg/l Kinetin, while the highest number of leaves of tetraploid plants found in MS media containing 1 mg/l Kinetin. Without addition of BAP or Kinetin the number of leaves in diploid and tetraploid plants are reduced. The number of leaves is influenced by the number of shoots formed, so the fewer shoots formed, the smaller number of leaves formed [51]. After eight weeks of cultures, performances of diploid and tetraploid plants are presented in Figure 5 and Figure 6.

The highest number of roots was found in diploid plants on MS media without growth regulators similarly on MS medium containing 0.5 mg/l Kinetin, and the lowest was on MS media containing 1 and 2 mg/l BAP (Table 2). The higher concentration of cytokinins added to the culture medium, the lower the amount of root formed. In general, root growth is influenced by auxin. The higher auxin concentration given to the medium gives the greater number of roots formed [52]. Research by Siregar [11] showed that the addition of 3 mg/l NAA to *P. amboinicus* increased the number of roots. According to Lestari [53] the addition of auxins or cytokinins can increase the concentration of endogenous growth regulator substances in the cell, so that it becomes a trigger factor for the process of tissue to grow and develop. This research is in line with Wulansari [47] that the higher the addition of Kinetin level, the less the number of roots in the Bolang and Pontianak taro plants [47]. Research by Mahadi [48] shows that NAA and Kinetin significantly affected the number of *Hylocereus costaricensis* roots.

3.2 Confirmation by flow cytometry analysis

Flow cytometry analysis of *P. amboinicus* is shown on Table 3 with the histogram is shown in Figure 7. The result indicated that the peak size for diploid plants was at the 80.279 channel and tetraploid was at the 159.547 channel with a diversity coefficient (CV%) ranged from 4.8 to 6.4%. Tetraploid plants have a PI value twice than that of diploid plants. The purpose of this flow cytometer analysis was to reconfirm the ploidy level after Kinetin and BAP treatment. The results showed that after the treatments all plants had stable in their ploidy levels.

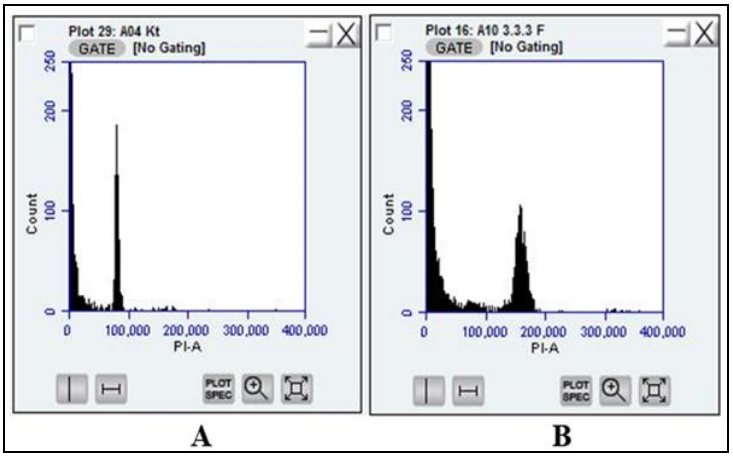

Figure 7. Histogram of diploid (A) and tetraploid (B) *Plectranthus amboinicus in vitro* plantlets at eight weeks from flow cytometry analysis.

3.3 Acclimatization

The acclimatization stage is an important stage in micropropagation. Acclimatization is the adaptation of changes in environmental conditions to grow gradually from *in vitro* to *en vitro* environment. Plantlet conditions must be vigorous in order to be able to survive in an *en vitro* environment, so acclimatization methods must be chosen according to the plant species [54]. Our results showed that

both plantlets *P. amboinicus* diploid and tetraploid had survival rate of 95% (one out of 20 plants died), even though the diploid plants were smaller than the tetraploid plants (Figure 8). The morphology of the *P. amboinicus* plant 12 weeks after acclimatization is shown in Figure 8. This study indicated that *P. amboinicus* tetraploid plants have higher productivity than diploid plants because they have a larger size.

Table 3. Mean PI and CV of *Plectranthus amboinicus* from flow cytometry analysis.

No	Clone	Mean PI	CV %	Ploidy level
1	Diploid	80.279	4.8	Diploid
2	Tetraploid	159.547	6.4	Tetraploid

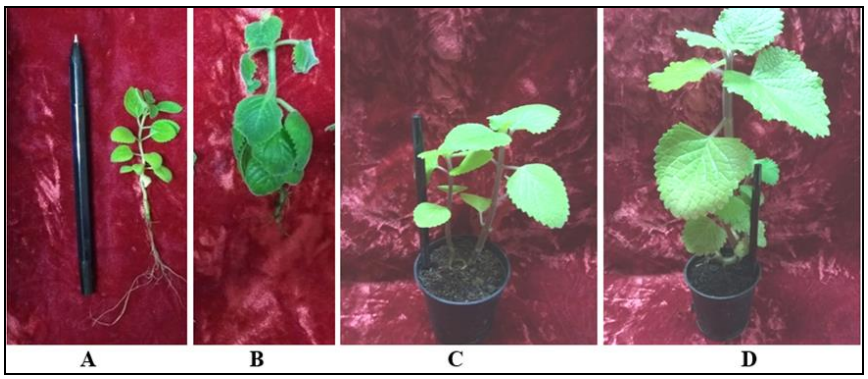

Figure 8. Diploid and tetraploid *Plectranthus amboinicus* grown in greenhouse at eight weeks (A-B) and 12 weeks (C-D) after acclimatization.

The results showed that both diploid *in vitro* plantlets and *en vitro P. amboinicus* plants had smaller stem diameters and lower leaf area compared to tetraploid plants (Table 4, Figure 9). The average leaf area of *P. amboinicus* tetraploid plantlets was approximately three times larger than the leaf area of diploid plantlets, while the leaf area of tetraploid *en vitro* plants was more than five times higher than diploid *en vitro* plants (Table 5). The results indicated that *P. amboinicus* tetraploid plants have higher productivity compared to diploid plants because of the larger leaf area. The bigger diameter of stem and the older plants shows the greater plant productivity [55]. In some species, tetraploid plants have biomass, size of fruit, bulbs or flowers that are larger than the diploid plants [50]. According to Sutoro and Setyowati [56] leaf area is an important plant character to study agronomic and physiological aspects. Leaf area is one of the variables to determine the vegetative growth of plants [56]. The importance of leaf area measurement is to study the photosynthesis process, and to determine the yield [57], to determine number of stomata and amount of chlorophyll [58], as well as to investigate heavy metal accumulation in leaves [59].

Table 4. Shoot diameter and leaf area of diploid and tetraploid *Plectranthus amboinicus* plantlets *in vitro* cultured on MS medium without plant growth regulators and *en vitro* plants at 12 weeks after acclimatization.

No	Clone	Stem diameter (cm)		Leaf area (mm^2)	
		In vitro	*En vitro*	*In vitro*	*En vitro*
1	Diploid	1.19^b	4.83^b	203.35^b	984.48^b
2	Tetraploid	2.16^a	6.83^a	723.95^a	$5,436.33^a$

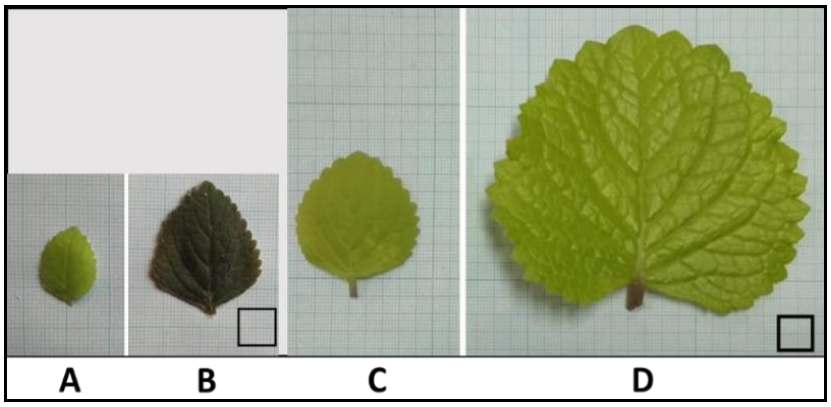

Figure 9. The leaf of *in vitro* diploid and tetraploid plantlets of *Plectranthus amboinicus* at eight weeks after planting (A-B) and *en vitro* diploid and tetraploid plants at twelve weeks after acclimatization (C-D). (Black square = 1 cm^2).

4. Conclusion

Both *P. amboinicus* diploid and tetraploid had better growth on medium containing Kinetin compared with BAP. Each growth and development of this plant required different type and level of cytokinins both for diploid and tetraploid plants. *In vitro* and *en vitro* tetraploid plants had a bigger stem diameter also bigger leaf size than that of diploid plants. *Plectranthus amboinicus* plants had high survival rate when it was acclimatized on mixed soil, compost and roasted media and kept in the greenhouse. Detection of chemical compounds in beneficial to find out the differences between diploid and tetraploid plants.

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